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An Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS) Method for the Rapid and Sensitive Determination of Sulforaphane and Sulforaphane Nitrile in Brassica Vegetables

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An Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method for the rapid and sensitive determination of sulforaphane and sulforaphane nitrile in *Brassica* vegetables

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Abstract

A rapid UPLC-MS/MS method has been developed and validated for the simultaneous analysis of sulforaphane and sulforaphane nitrile from *Brassica Oleracea* L. This method was developed utilising an Acquity BEH C8 column with gradient elution combined with tandem mass spectrometry detection, using positive ion electrospray ionisation in multiple reaction monitoring (MRM) mode. The method was validated for linearity, sensitivity, precision, accuracy, matrix effects and recovery. The retention times for sulforaphane and sulforaphane nitrile were 0.4 and 0.6 min, respectively. The limits of detection and lower limits of quantification were 0.005 μM and 0.01 μM respectively for sulforaphane, and 0.06 μM and 0.16 μM for sulforaphane nitrile respectively. Intra- and inter-day precision ranged, respectively, from 5.07 to 8.93 % RSD and from 3.44 to 9.31 % RSD, for sulforaphane and from 0.95 to 6.69 % RSD and 6.84 to 9.94 % RSD, respectively, for sulforaphane nitrile. The accuracy varied from -12.55 to 10.34 % bias for sulforaphane and from -8.67 to 8.06 % bias for sulforaphane nitrile. Matrix effects were also investigated and were found to be in the range of 92.5 to 105.7 %, and 99.2 to 102.9 %, for sulforaphane and sulforaphane nitrile respectively. This method is the first validated UPLC-MS/MS method for the rapid and sensitive determination of sulforaphane and sulforaphane nitrile in cruciferous sources such as broccoli plants.

Keywords:

Sulforaphane, *Brassica*, Ultra-Performance Liquid Chromatography (UPLC), Mass Spectrometry (MS), Multiple reaction monitoring (MRM)

1. Introduction

Sulforaphane (SFN) is the common name for 4-methylsulphinylbutyl isothiocyanate. Isothiocyanates such as sulforaphane are derived from the enzymatic hydrolysis of glucosinolates (β -D-thioglucose linked to a sulfonated oxime and a variable side chain), secondary metabolites found in cruciferous vegetables. The enzyme responsible for this hydrolysis is an endogenous thioglucosidase enzyme named myrosinase (EC 3.2.3.1). Cruciferous vegetables generally contain a range of different glucosinolates, however, *Brassica Oleracea L var Italica* (broccoli) is particularly rich in glucoraphanin, the glucosinolate precursor of sulforaphane [1, 2].

Glucosinolates in plant tissues are physically separated from myrosinases [3]. Therefore, when plant tissue containing glucosinolates is disrupted (blending, cutting or chewing) the endogenous enzyme myrosinase is able to hydrolyze glucosinolates into isothiocyanates [3]. The enzymatic hydrolysis leads to glucose and an unstable aglycone (thiohydroxamate -O-sulfonate) that can rearrange spontaneously to form different possible products. Therefore, in addition to isothiocyanates, nitriles are also formed, as well as thiocyanates and epithionitriles to a smaller degree [4]. The type and ratio of the hydrolysis product depends on the particular side chain of the glucosinolate and other reaction conditions such as temperature, pH, ascorbic acid content and Fe^{2+} ions [4]. An important factor also known to affect the type and ratio of the hydrolysis products from the glucosinolate-myrosinase system is a supplementary protein named epithiospecifier protein (ESP). ESP is a myrosinase co-factor that has been shown to direct the myrosinase-dependent hydrolysis of glucoraphanin to form sulforaphane nitrile in place of the isothiocyanate sulforaphane [5]. This finding is of great importance since sulforaphane has been shown to have bioactivity as a cancer prevention agent whereas sulforaphane nitrile (SFNN) is largely inactive [6]. Sulforaphane modes of action are well documented in the literature and have been reviewed elsewhere [7-9]. In brief, SFN modulates many cancer-related events, including susceptibility to carcinogens, cell death, cell cycle, angiogenesis, invasion and metastasis [7]. In addition, sulforaphane has also been shown to have anti-inflammatory effects thereby inhibiting cytokine production [8]. Therefore, the interest in the SFN content of foods has consequently increased notably. It is also important to note that the potential health benefits of cruciferous vegetables may be increased by promoting the formation of SFN production over SFNN by attenuating ESP activity. Previous studies have demonstrated that ESP

activity differs depending on the type of *Brassica* species [10], and is affected by several factors including the presence of Fe^{2+} [10] and also heating has been shown to decrease ESP activity [11].

Several analytical methods are currently employed for the determination of SFN. UV spectrophotometric quantification of isothiocyanates is widely employed and is based on the cyclocondensation of the isothiocyanate group with 1,2-benzenedithiol, to produce a cyclic condensation product, 1,3-benzodithiole-2-thione with maximum absorbance at 365 nm. This direct and generic method is commonly used because it is easy to implement. However its sensitivity is somewhat low (1.5 μM and 4.5 μM limit of detection and limit of quantitation respectively). A subsequent study demonstrated that the sensitivity of the method may be improved by separating the cyclocondensation product with an isocratic HPLC method [12]. It is important to note that this method is not specific to sulforaphane since the chemical specificity of the cyclocondensation reaction is not restricted to sulforaphane and includes other isothiocyanates present in the sample as well as dithiocarbamates and related thiocarbonyl compounds.

Several HPLC methods have also been employed for the determination of SFN. In these studies detection is conducted by monitoring UV absorbance at a particular wavelength which was found to vary depending on the study (i.e. 235, 254 and 202 nm). The detection time for sulforaphane ranged from 5.68 to 14.18 min and the run time was in the range of 10 to 20 min [13-15]. GC-MS has also been used for the analysis of sulforaphane and sulforaphane nitrile in *Brassica* vegetables. GC-MS offers increased sensitivity over HPLC methods however sample preparation may involve elaborate and time consuming derivatization procedures. Chiang et al. [16] developed a GC-MS method in which thermal degradation of sulforaphane was reduced to 5% through the use of an appropriate injector liner and precise control of the carrier gas flow rates. However, the total run time for this method was of 25 minutes. Limit of detection was reported to be 2 $\mu\text{g/g}$ fresh weight for both sulforaphane and sulforaphane nitrile.

In recent years more sensitive and specific methods based on LC-MS have been developed. Song, Morrison, Botting, & Thornalley [17] analyzed glucosinolates and isothiocyanates including sulforaphane in vegetable extracts and blood plasma by LC-

MS/MS. Isothiocyanates were prederivatized to thiourea to achieve high sensitivity levels (0.5 – 2 pmol). However, recoveries of isothiocyanates in this method were in the range 50-85 %. Agrawal, Winnik, Buckley, Mi, Chung, & Cook [18] developed a sensitive and specific liquid chromatography-tandem mass spectroscopy method for the determination of SFN and its major metabolites from biological matrices. The limit of quantification for sulforaphane from rat plasma was 0.02 μ M. However, the retention time for SFN was 28.2 minutes. Also Al Janobi *et al* developed a quantitative LC-MS method for the determination of sulforaphane, iberin and their metabolites in human urine and plasma. Gradient liquid chromatographic separation was performed using similar chromatographic conditions to those by Agrawal and co-workers. The limit of quantification for SFN in human plasma was 0.015 μ M, and SFN had a retention time of 9.1 minutes [19].

Several analytical methods exist therefore for the determination of sulforaphane and sulforaphane nitrile from biological matrices and plant extracts. However, no rapid and sensitive method exists for the simultaneous detection of sulforaphane and sulforaphane nitrile in plant matrices. LC-MS/MS methods consisting of UPLC systems attached to a tandem quadrupole detector (TQD) offer several advantages in particular when rapid, sensitive and specific analytical methods are required. UPLC systems are characterized by greatly reduced run times over conventional LC systems. Also, TQDs are characterised by their ease of use compared to quadrupole-time-of-flight mass detectors, and their cost is significantly lower. As a result, TQDs are currently standard quantification mass detectors in the food industry and UPLC systems attached to a TQD are particularly important in applications where high through-put, low cost and ease-of-use are required.

The rapid and reliable monitoring of glucosinolate hydrolysis products in plants and plant-based food products is of great relevance since sulforaphane and sulforaphane nitrile differ greatly in their bioactivity [6] and has many important applications. It may be employed for the study of the factors governing the conversion of glucosinolates by myrosinase and the production of SFN over SFNN. It may therefore be employed in the optimisation of reaction conditions to direct the conversion towards the formation of bioactive sulforaphane over the largely inactive sulforaphane nitrile [6] by controlling processing factors such as temperature, pH, processing time and ingredient interactions.

Another direct application includes the study of the stability of SFN and SFNN during processing as well as during storage, and as a high through-put method for the rapid screening of different varieties and cultivars, as well as the assessment of the effect of agronomic and environmental factors on the glucosinolate-myrosinase system. The aim of this study was therefore to develop and validate a specific, sensitive and rapid UPLC-MS/MS method using MRM for the simultaneous determination of sulforaphane and sulforaphane nitrile in plant and plant-based food extracts.

2. Experimental

2.1 Chemicals

LC-MS grade acetonitrile (CH₃CN), mass spectrometry grade ammonium acetate and formic acid, along with magnesium sulphate, sodium chloride, DL-sulforaphane (90% purity), and HPLC-grade water were obtained from Sigma-Aldrich (Arklow, Ireland). Sulforaphane nitrile standard was isolated and purified from broccoli seeds as described in the section below. Broccoli seeds were supplied from a local store (Nourish, Dublin, Ireland).

2.2 Isolation and purification of sulforaphane nitrile

Sulforaphane nitrile was extracted from broccoli seeds according with the method previously published by Matusheski, Wallig, Juvik, Klein, Kushad, & Jeffery [20] with minor modifications. Briefly, 50 g of broccoli seeds were ground to a fine powder using a metal ball mill (Retsch MM 400, Retsch Ltd, Castleford, UK). Ground seed was then defatted 3 times with excess hexane and left to air dry overnight in a fume hood. Deionised water was then added to the ground seed in a ratio of 3:1 water/defatted seed (w/w), and the mixture was allowed to autolyse for 8 hours at room temperature to promote glucosinolate enzymatic hydrolysis. Sodium chloride and sodium sulphate were added to the seed autolysate in the ratio of 1:0.75:1 (w/w/w) and the resultant paste was extracted with excess methylene chloride. Extraction was repeated two more times and the methylene chloride layers were combined. Magnesium sulphate was added to the methylene chloride extract to remove traces of water, this was then filtered through a Buchner funnel and dried under vacuum at 32 °C using a rotatory evaporator (Heidolph Laborota 4000 efficient, Heidolph Instruments GmbH & Co KG, Schwabach, Germany). The obtained residue was dissolved in 5 % acetonitrile in water (v/v) (12 mL) and filtered using 0.22-µm pore size PVDF membrane filters (PALL Life Sciences, USA) in preparation for injection onto preparative HPLC. Purification of sulforaphane nitrile was performed on a preparative HPLC system (Varian Pro Star, Varian, Inc, Walnut Creek, USA) using a Phenomenex Luna (100 x 21.20 mm, 100 Å, 5 µ) C-18 reversed-phase HPLC column (Phenomenex, Inc, Chesire, UK). The mobile phase consisted of ammonium acetate buffer (10 mM, pH = 4.5) (Mobile phase A) and acetonitrile (Mobile phase B) and flow rate was 10 mL/min. The gradient started at 5 % (Mobile phase B), held for 10 min at 5 % B, increased to 10 % B over 2 min, then to 20 % B over 8 min, with a further increase to 90 % B over 10 min to purge the column,

finally re-equilibrated to 5 % B over 2 min and maintained 3 min. Injection volume was 4 mL. Since sulforaphane nitrile does not absorb strongly in the UV, sulforaphane was used as a reference to identify which preparative-HPLC fractions may contain sulforaphane nitrile. Sulforaphane nitrile is more polar compared to SFN and therefore its retention time is shorter. Sulforaphane was detected using a Varian ProStar 335 photodiode array detector (Varian, Inc, Walnut Creek, USA) at 254 nm in fraction 9 with elution times between 16 and 18 min. All fractions were collected and those fractions eluting previous to sulforaphane were concentrated using a nitrogen drier (Techne DB-3, Bibby Scientific Ltd, Staffordshire, UK) and analysed by LC-ESI-MS/MS for sulforaphane nitrile. LC-MS analysis was conducted using a Q-ToF Premier mass spectrometer (Waters Corporation, Micromass MS Technologies, Manchester, UK) attached to Alliance 2695 HPLC system (Waters Corporation, Milford, MA, USA). The internal reference compound (Leucine-Enkephalin) was introduced simultaneously with the analyte through the lockspray source for accurate mass measurements. Separation was conducted on an Atlantis T3 C18 column (Waters Corporation, Milford, USA, 100 mm x 2.1 mm; 3 μ m particle size). Column temperature was maintained at 40 °C. The mobile phase consisted of ammonium acetate buffer (10 mM, pH = 4.5) (solvent A) and 0.1% formic acid in acetonitrile (solvent B) and flow rate was 0.2 mL/min was used throughout. A stepwise gradient from 10 % to 90 % solvent B was applied over 26 min. Mass spectral data was obtained in positive mode with a mass range of m/z 100 to m/z 1000. Capillary voltage and cone voltage were set at 3 kV and 30 V respectively. Collision induced fragmentation (CID) of the analytes was achieved in MSe mode using 12 eV to 20 eV energy with helium as the collision gas. Sulforaphane nitrile was detected in fraction 5 with elution times between 8 and 10 min.

2.3 Sample preparation

Lyophilized and finely milled broccoli samples (50 mg) were weighed and water was added in a ratio of seed to water of 1:10 (w/v). Samples were autolysed for 30 minutes at room temperature to promote glucosinolate hydrolysis. Samples were then extracted by liquid-liquid partitioning using ethyl acetate (3 mL). Magnesium sulphate was also added to the autolysate mix to obtain a saturated solution and therefore to facilitate the extraction of sulforaphane and sulforaphane nitrile into the ethyl acetate phase. Samples were then vortexed for 20 min at 2000 rpm (Heidolph Multi Reax, Heidolph Instruments GmbH & Co KG, Schwabach, Germany) and centrifuged for 5 min at 8603

g (SIGMA 2-16 PK, SIGMA Laborzentrifugen GmbH, Osterode am Harz, Germany). The ethyl acetate layer was then transferred to a clean test tube and the remaining aqueous phase was re-extracted with ethyl acetate as described above. This process was repeated one more time. The ethyl acetate layers were combined and dried under nitrogen. The resultant residue was then re-suspended in 6 mL of initial conditions mobile phase (70 % ammonium acetate buffer (10 mM, pH = 4.5): 30 % acetonitrile with 0.1 % formic acid), filtered through a 0.22 µm pore size PVDF membrane filters (PALL Life Sciences, USA) and stored at -40 °C prior to analysis.

2.4 UPLC-MS conditions

Chromatographic experiments were carried out on a Water Acquity UPLC system managed by Acquity console software (Waters Corp., Milford, USA). Separation was achieved on a Waters Acquity BEH C8 column (1.7 µm, 2.1 x 50 mm) equipped with a Waters C8 VanGuard (5mm x 2.1 mm; 1.8 µm particle size). The column oven temperature was maintained at 40 °C and the autosampler at 4 °C. The mobile phase consisted of ammonium acetate buffer (10 mM, pH = 4.5) (Mobile phase A) and 0.1% formic acid in acetonitrile (Mobile phase B) and a flow rate of 0.5 mL/min and an injection volume of 5 µL were used throughout. The gradient conditions initially started at 30 % (Mobile phase B) increasing over 1.5 min to 90 % B, held for 1 min at 90 % B and finally re-equilibrated to 30 % B over 0.5 min.

The UPLC system was attached to a triple quadrupole mass spectrometer (Waters Acquity TQD, Waters Corp., Milford, USA). The instrument was operated in the ESI positive mode. The source temperature was set at 120 °C, desolvation temperature was fixed at 350 °C. Capillary voltage was set at 3 kV and cone voltage was optimised for each of the compounds by using IntelliStart™ software (Waters Corp., Milford, USA). Nitrogen gas was used as both sheath gas and auxiliary gas (800 L/hr and 50 L/hr respectively). Detection of the analytes was conducted in the multiple reaction monitoring (MRM) mode by analysing two transition ions per compound. The MRM conditions were determined and optimised by tuning both sulforaphane and sulforaphane nitrile using Waters integrated IntelliStart™ software (Waters Corp., Milford, USA).

2.5 Validation

The developed method was subsequently validated for sensitivity, linearity (R^2), intra- and inter-batch precision, accuracy and recovery according to the requirements of USFDA [21]. Matrix effects were evaluated in accordance with the method of Matuszewski et al. [22] Sensitivity was assessed by means of limit of detection (LOD) and lowest limit of quantitation (LLOQ) values. Furthermore, intra- and inter-day precision were determined at the obtained LLOQ values. Precision, accuracy, matrix effect and recovery were evaluated using the method of standard addition. Precision and accuracy were assessed at three different concentrations (i.e. low at 0.1 and 0.3 μM ; medium at 2.0 and 1.7 μM and high at 5.0 and 5.7 μM for sulforaphane and sulforaphane nitrile respectively). Intra-day precision was evaluated by calculating the relative standard deviation (RSD) of 5 determinations per concentration in a single run in the case of low and medium concentration samples, and three determinations in a single run for high concentration samples. Inter-day precision was calculated using three determinations per concentration on three different days. In addition, accuracy was evaluated by replicate analysis of spiked samples (5 determinations per low, medium, and high concentration) on a single run. According to the recommendations published by the USFDA, the criteria for data acceptability establish that precision should be within $\pm 15\%$ RSD, except at the lowest limit of quantitation where precision should be within $\pm 20\%$ of RSD. Accuracy should not exceed $\pm 15\%$ deviation (or bias) from the nominal value [21]. Recovery for each of the two compounds was calculated by spiking the broccoli extract with standard working solutions. Matrix effect was also evaluated and was calculated as $(B-C)/A \times 100$ with (A) being the peak area of neat standards in initial mobile phase, (B) the peak area of a broccoli matrix spiked with the same amount of standards and (C) the peak area of endogenous amounts of sulforaphane and sulforaphane nitrile present in the broccoli matrix [22].

3. Results and Discussion

3.1 UPLC-MS/MS

The mobile phase employed was chosen based on previously published results by Agrawal, Winnik, Buckley, Mi, Chung, & Cook [18] in which ammonium acetate buffer exhibited optimal ionisation of the analyte sulforaphane. Chromatographic conditions were optimized for resolution, increase analyte signal, and reduced run times with no sample carryout between injections by changing the pH of the buffer and gradient conditions. A greatly reduced run time is one of the major characteristic features of UPLC. This is achieved by the use of smaller particle size columns capable

of higher back pressure limits in comparison with conventional HPLC columns, resulting in reduced analysis times, as well as increased peak capacity and resolution. [18]. This results in the ability to resolve analytes with shorter retention times along with enhanced peak shape and sensitivity. Selective reaction monitoring mode was used for the determination of sulforaphane and sulforaphane nitrile, resulting in improved assay selectivity by detecting and quantifying compound-specific fragments obtained by CID and eliminating interferences from other constituents of the sample [18]. Positive ionisation mode was selected for sulforaphane and sulforaphane nitrile analysis due to the chemical structures of the analytes and their poor ionisation behaviour observed in negative ion mode (REF). Multiple reaction monitoring (MRM) transitions and compound parameters for sulforaphane and sulforaphane nitrile are presented in Table 1. As illustrated in Figure 1, sulforaphane during CID undergoes cleavage at sulfinyl-carbon bond resulting in the formation of isothiocyanato-butene which cyclises to form a stable product ion m/z 114 [23]. The formation of the product ion m/z 55 can be attributed to the loss of ionic thiocyanate [23]. The retention times for sulforaphane nitrile and sulforaphane were 0.4 and 0.6 min respectively. The UPLC-MS/MS chromatograms of an autolyzed sample of *Brassica Oleracea* L with MRM transitions for sulforaphane and sulforaphane nitrile are represented in Figure 2.

3.2 Method validation

The results obtained for the validation of the linearity of the proposed method are presented in Table 2. Limit of detection (LOD) and lowest limit of quantitation (LLOQ) for sulforaphane and sulforaphane nitrile were calculated by determining the concentrations of sulforaphane and sulforaphane nitrile standards that resulted in defined mass spectral peaks with signal-to-noise ratios of 3 and 10, respectively. The limits of detection and lowest limits of quantification were 0.005 μM and 0.01 μM respectively for sulforaphane, and 0.06 μM and 0.16 μM for sulforaphane nitrile, respectively. The calibration curves for sulforaphane and sulforaphane nitrile were constructed using seven calibration samples covering the range of 0.01 to 5.08 μM for sulforaphane, and six calibrators in the range of 0.16 to 6.2 μM for sulforaphane nitrile. Calibration curves were calculated by linear regression using $1/X$ as a weighing factor. Linearity was assessed by the coefficient of determination (R^2) which was in all cases higher than 0.997 for both compounds. Intra- and inter-day precisions were also estimated at the limit of quantification levels. The obtained intra- and, inter-day RSD

(%) was lower than 16 % for both sulforaphane and sulforaphane nitrile (Table 2), which is within the acceptable range of 20 % at the LLOQ.

The precision and accuracy data for the determination of sulforaphane and sulforaphane nitrile in the test samples are presented in Table 3. Intra-day precisions at all three concentration level tested (low, medium, and high) were in the range from 5.07 to 8.93 % in the case of sulforaphane and from 0.95 to 6.69 % for sulforaphane nitrile. The inter-day precisions were also within the acceptable range of 15 % at concentrations above the lowest limit of quantitation. In particular, inter-day precisions ranged from 3.44 to 9.31 % for sulforaphane and from 6.84 to 6.99 % in the case of sulforaphane nitrile. Acceptable accuracies (bias < 15%) were obtained at all three levels of concentration (low, medium, and high) for both sulforaphane and sulforaphane nitrile. In particular, accuracy was found to vary from -12.55 to 10.34 % bias for sulforaphane, and from -8.67 to 8.06 % bias for sulforaphane nitrile.

The matrix effect of the newly developed method was evaluated by spiking broccoli extracts across the linearity range and comparing the results with those of pure standards of the same concentrations. It is essential to determine and demonstrate low matrix effects in mass spectrometry as co-eluting matrix compounds can compete with the analytes of interest for the surface of the solvent droplet, enhancing or suppressing ion intensity and therefore affecting both selectivity and reliability of the assay [22]. Matrix effect was calculated across the linear range and was found to be in the range of 92.5 to 105.7 %, and 99.2 to 102.9 %, for sulforaphane and sulforaphane nitrile respectively, therefore indicating minimal matrix effect. These results are shown in Table 3. Recovery for each of the two compounds was calculated by spiking the broccoli extract with standard working solutions of concentrations equivalent to the endogenous levels in the broccoli extracts. Two different broccoli cultivar extracts were employed in the recovery experiments both containing different ratios of sulforaphane to sulforaphane nitrile. Broccoli powder from Ironman cultivar had a higher sulforaphane to sulforaphane nitrile ratio compared to broccoli powder from Marathon cultivar. Ironman and Marathon recovery samples were prepared in triplicate on separate days and analysed on two separate runs. These results are presented in Table 4. The mean extraction recovery of SFN and SFNN from Marathon was 101.8 and 97.0 %, respectively, whereas from Ironman recovery was 96.2 and 113.4 %, for SFN and

SFNN respectively. Recoveries were also reproducible and variation in all cases was always lower than 8 % (RSD).

3.3 Application of the developed UPLC-MS/MS method

The validated method has been applied successfully for the quantitation of sulforaphane and sulforaphane nitrile in broccoli samples. Sulforaphane and sulforaphane nitrile concentrations in two different broccoli varieties are presented in Table 4. It can be observed that the predominant glucoraphanin hydrolysis product varied depending on the variety tested. Sulforaphane was predominant in Ironman whereas sulforaphane nitrile was the major product in the case of Marathon broccoli. Different results can also be observed in the literature and it has been showed that the formation of one hydrolysis product over the other may depend on factors such as genotype [5]. In addition, the formation of sulforaphane and sulforaphane nitrile is also known to be influenced by the reaction conditions including the temperature of the reaction [11] and the presence of Fe^{2+} ions [10]. In the present study, the validated method was also applied to the accurate determination of the levels of sulforaphane and sulforaphane nitrile in broccoli samples hydrolysed under different conditions. These results are presented in Table 5. Different levels of sulforaphane and sulforaphane nitrile were measured and the ratio of sulforaphane to sulforaphane nitrile was shown to vary according with the hydrolysis conditions.

Considering that sulforaphane nitrile is known to be substantially less potent than sulforaphane as an inducing agent of phase II detoxification enzymes [6], it is important to characterize the products of glucoraphanin hydrolysis. Directing glucoraphanin hydrolysis toward the production of sulforaphane over sulforaphane nitrile could increase the potential chemoprotective effects of broccoli [6].

4. Conclusion

The new UPLC-MS/MS method presented in this study for the simultaneous quantitation of sulforaphane and sulforaphane nitrile from broccoli has been developed and validated. The method is linear in the range of 0.01 to 5.08 μM for sulforaphane, and 0.16 to 6.2 μM for sulforaphane nitrile and validation experiments demonstrated that both accuracy and precision were acceptable within the linear range. Validation experiments also demonstrated minimal matrix effects across the linear range. This method is sensitive with LLOQ of 0.01 μM and 0.16 μM for sulforaphane and

sulforaphane nitrile respectively. Moreover, this method provides significantly shorter analysis times (3 min) compared with previously reported analytical methods. The short analysis time of the newly developed method represents a significant advantage particularly when high throughput is required. For instance, this method may be employed in the optimisation of critical factors during the processing and storage of brassica plants and brassica-based food products to obtain maximum levels of the bioactive SFN. In addition, this type of method will allow the rapid screening of varieties and cultivars to assess which brassicas represent important sources of the bioactive SFN, as well as to ascertain the influence of climatic and agronomic factors on the glucosinolate-myrosinase system and the production of SFN and SFNN. In the present study, the developed method was successfully applied for analysis of broccoli samples to study key glucoraphanin hydrolysis products with important biological activity. To conclude, the newly developed UPLC-MS/MS method is a simple and reliable analytical method for the simultaneous determination of sulforaphane and sulforaphane nitrile in broccoli with high sensitivity and short analysis times.

1 **Table 1.** Multiple reaction monitoring (MRM) transitions and compound parameters for
 2 sulforaphane and sulforaphane nitrile on positive ion mode.

3

	Precursor ion (m/z)	Product ion (m/z)	Cone (V)	Collision (eV)
Sulforaphane	178.0	114.0	24	30
	178.0	55.0	24	10
Sulforaphane	146.1	55.0	26	20
nitrile	146.1	81.9	26	14

4

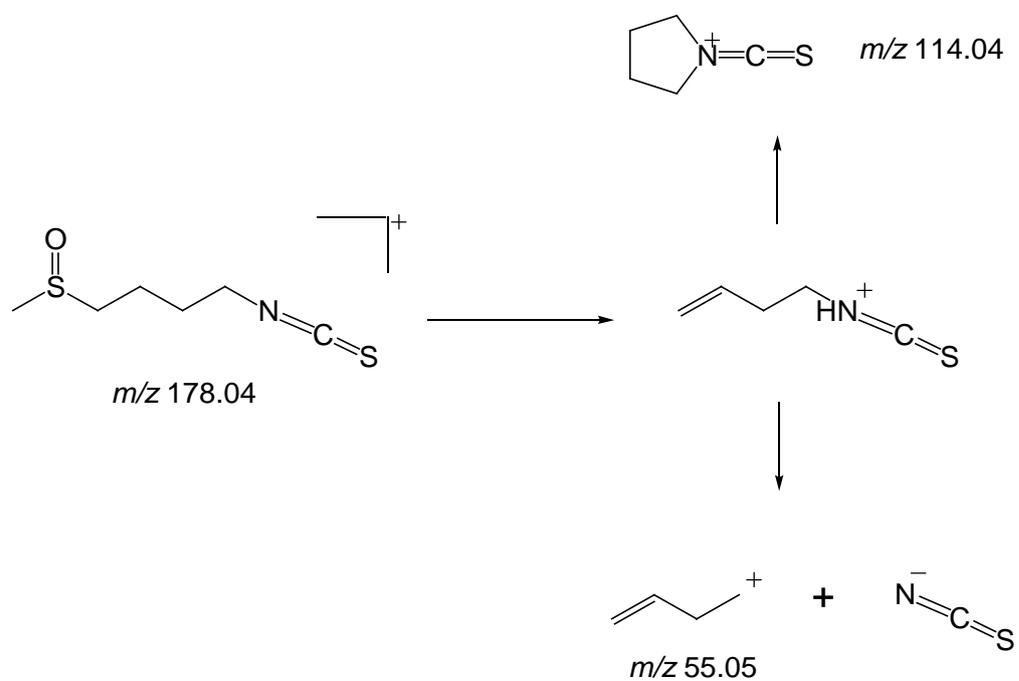
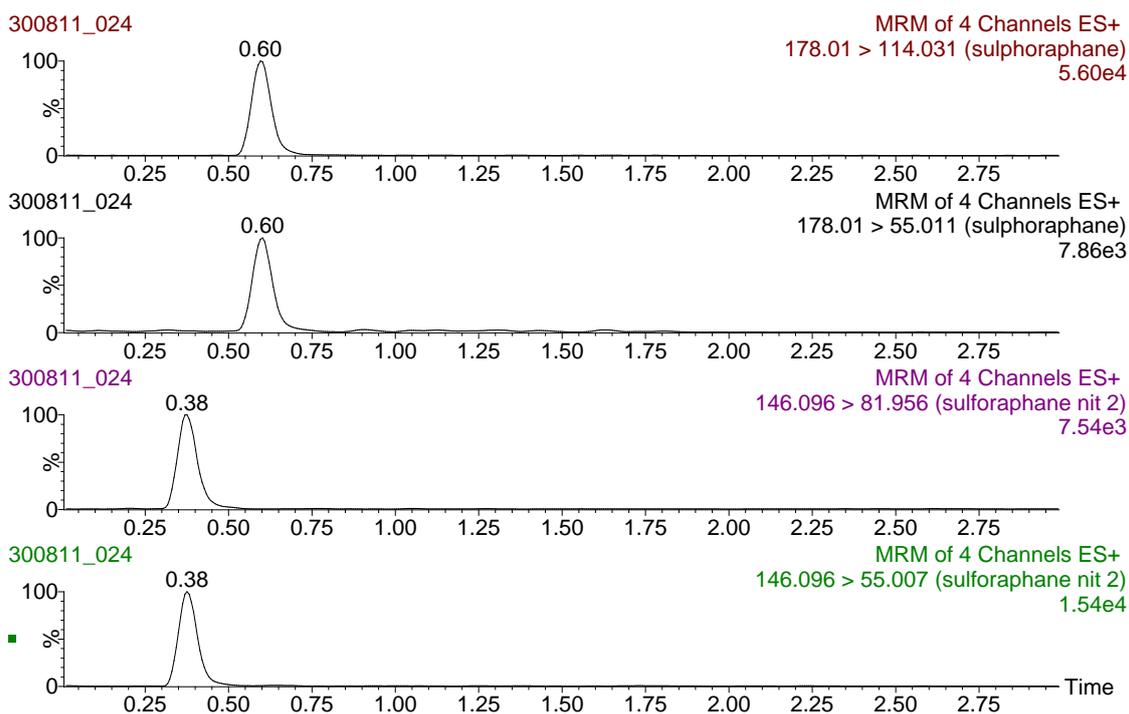


Figure 1. Multiple reaction monitoring (MRM) transitions for sulforaphane on positive ion mode

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13

14 **Figure 2.** UPLC-MS/MS chromatogram with MRM transitions for sulforaphane and
15 sulforaphane nitrile of an autolyzed sample of *Brassica Oleracea* L. The
16 chromatographic conditions employed are described in the experimental section.

17

18 **Table 2.** Calibration results of the proposed method for sulforaphane and sulforaphane
 19 nitrile in broccoli.

20

	LOD (μM)	LLOQ (μM)	Slope (n=3) mean \pm SD	Intercept (n=3) mean \pm SD	R^2	Linear range (μM)	Intra-day precision (CV %) at LLOQ n=5	Inter-day precision (CV %) at LLOQ n=4
Sulforaphane ($\text{C}_6\text{H}_{11}\text{NOS}_2$)	0.005	0.01	105252.83 \pm 8607.59	83.19 \pm 122.86	0.9987	0.01 - 5.08	11.18	15.45
Sulforaphane nitrile ($\text{C}_6\text{H}_{11}\text{NOS}$)	0.06	0.16	6521.64 \pm 1078.88	16.82 \pm 25.34	0.9978	0.16 - 6.2	12.41	12.27

21

22

23

24

25 **Table 3.** Precision, accuracy and matrix effect data for the determination of
 26 sulforaphane and sulforaphane nitrile in brassica vegetables.

Validation parameter	Sulforaphane (C ₆ H ₁₁ NOS ₂)			Sulforaphane nitrile (C ₆ H ₁₁ NOS)		
	Low	Medium	High	Low	Medium	High
Intra-day precision (% CV) n=5 (low, medium), n=3 (high)	8.93	5.96	5.07	6.69	4.19	0.95
Inter-day precision (% CV) n=3	9.31	3.69	3.44	6.99	6.84	9.94
Accuracy (% bias) n=5	-10.4 to 10.3	0.5 to 5.3	-12.6 to - 0.1	-2.9 to 8.1	-0.2 to 7.6	-8.7 to 5.2
Matrix effect (%) n=5	105.7 ± 11.7	103.2 ± 3.3	92.5 ± 5.1	102.9 ± 6.3	104.1 ± 3.8	99.2 ± 5.4

27

28

29 Table 4. Extraction recovery results of sulforaphane and sulforaphane nitrile from
 30 Marathon and Ironman broccoli freeze-dried powders.

31

	Marathon broccoli		Ironman broccoli	
	Sulforaphane	Sulforaphane nitrile	Sulforaphane	Sulforaphane nitrile
Endogenous concentration (μmol/g)	0.30 ± 0.01	1.73 ± 0.05	3.26 ± 0.14	1.30 ± 0.03
Recovery (%)	101.8	97.0	96.2	113.4
RSD (n=3)	3.7	8.0	5.1	3.4

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	Marathon broccoli		Ironman broccoli	
	Sulforaphane	Sulforaphane nitrile	Sulforaphane	Sulforaphane nitrile
Endogenous concentration (μg/g)	54.0 ± 5.56	250.92 ± 7.98	576.60 ± 25.23	189.0 ± 3.98
Recovery (%)	101.8	97.0	96.2	113.4
RSD (n=3)	3.7	8.0	5.1	3.4

34

35 Table 5. Sulforaphane and sulforaphane nitrile content (ug/g dwb) in Ironman broccoli
 36 following hydrolysis under different conditions.

37

	High	Medium	Low
Incubation	40 °C, 60 min	None	90 °C, 60 min
Autolysis	Room temp, 60 min	Room temp, 60 min	Room temp, 60 min
Sulforaphane (µg/g dwb)	538.76 ± 47.76	454.68 ± 24.15	22.98 ± 0.92
Sulforaphane nitrile (µg/g dwb)	42.96 ± 0.49	65.89 ± 7.13	28.68 ± 0.36
Ratio (sulforaphane/sulforaphane nitrile)	12.54	6.9	0.8

38 Values displayed are means ± standard deviation (n=3)

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41

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